ANDROGENESIS, A DIFFERENTIATOR OF CYTOPLASMIC INJURY INDUCED BY X-RAYS IN HABROBRACON EGGS[1]

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Introduction

Any technic which differentiates cytoplasmic from chromosomal injury induced in the living cell by x-rays is of interest to investigators of biological effects of ionizing radiations. Injury to chromosomes is measured by their breakage and rearrangement and by visible and lethal mutations. Injury induced in the cytoplasm must, ordinarily, be studied by means of tests for physical and chemical changes in treated cells or by changes in behavior of the cell as a whole, inhibition of division, etc.

When evidence for the nature of effect is incomplete, it is always tempting to attribute greater sensitivity of egg than of sperm to cytoplasmic injury because of the extreme difference between these two types of cells in respect to amount of cytoplasm. Muller (1937) writes, "In Drosophila, a given irradiation of the eggs shortly before or after fertilization will result in non-development and death of a high proportion of them, whereas the same amount of treatment of spermatozoa alone allows more of the fertilized eggs to develop. Nevertheless genetic analysis of the resulting adults proves that they contain more genetic changes, *and more drastic ones,* in the latter case than in the former. The *difference* in death rate here, then, must have been of non-genetic origin, involving, no doubt, some injurious change in the egg protoplasm. On the other hand, the death of the embryos that were derived from treated sperm and untreated eggs must have been genetic." In experiments on irradiation of females of Drosophila, dose has not exceeded 6000 r.

Whiting, P. W. (1938) found that some eggs of Habrobracon survived a dose of 18,000 r (lethal dose for sperm in this species is about 10,000 r) and Whiting (1938) identified these as eggs irradiated in first meiotic prophase. Eggs treated in first meiotic metaphase were much more sensitive than sperm. This order in respect to size of lethal dose also holds for Sciara as reported by Metz and Boche (1939) and Reynolds (1941). In contrast to the results cited by Muller, then, eggs of Habrobracon and of Sciara are either more sensitive than sperm or less so according to condition of the chromosomes at time of treatment.

It is a generally accepted fact that the stage in the nuclear cycle is of importance in determining degree of sensitivity to x-rays and Muller in his epoch-making paper on artificial transmutation of the gene (1927) writes, "In addition, it was also pos-

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sible to obtain evidence in these experiments for the first time, of the occurrence of dominant lethal genetic changes, both in the X and in the other chromosomes. Since the zygotes receiving these never develop to maturity such lethals could not be detected individually but their number was so great that through egg counts and effects on the sex ratio evidence could be obtained of them en masse." This refers to dominant lethal changes induced in the sperm.

There is no argument, *a priori,* for the supposition that sperm have a monopoly on dominant lethal chromosome effects. The treatment of eggs in a nuclear stage which responds to relatively low doses of x-rays by the production of dominant lethal genetic changes will explain the greater sensitivity of eggs than of sperm.

The "genetic analysis of the resulting adults" to which Muller refers is an analysis of recessive or of non-lethal changes. Dominant lethal genetic or cytological effects are, of necessity, "strained out" in the process of reaching adulthood. As Sparrow point outs (in press), "In the presence of a low frequency of rejoining, an increased percentage of acentric fragments or deletions would be expected and thus a higher proportion of lethality would occur. Paradoxically, therefore, if one were scoring for aberrations in the F_1 generation, following radiation of one or both of the parental gonads, the recovered aberrations would not necessarily represent a true picture of total chromosome breakage since cells in the most sensitive stages would be the least likely to produce viable F_1's."

Habrobracon eggs vary greatly in their response to x-rays according to stage at time of treatment, and in the most sensitive stage studied (four times as sensitive as the sperm), cytological observation of large numbers of them after treatment has shown that death is due to chromosomal injury. The chromosomes of Habrobracon ($2n = 20$) are small, however, and any evidence for these conclusions gained from a different method of approach is of value and should help to convince those accustomed to work with large chromosomes who tend to be skeptical of observations made on small ones.

Androgenetic males, developed from the untreated sperm nucleus in x-rayed cytoplasm, furnish material for this different method of approach. A perfectly normal, fully fertile individual must, of necessity, have developed in an egg with cytoplasm not seriously altered by its exposure to x-rays.

Material and Methods

Wild type stock (No. 33) of the parasitic wasp, *Habrobracon juglandis,* with a hatchability of 96–98 per cent, has been used for all experiments since the beginning of this study in 1937.

Homozygous females of this stock were x-rayed and mated to untreated males with one or more traits recessive to wild type. These females were placed with host caterpillars, and their eggs were studied in respect to hatchability and/or were allowed to mature. Viable unfertilized eggs develop into wild type, gynogenetic males (both chromosomes and cytoplasm irradiated). Viable fertilized eggs develop into wild type, heterozygous, biparental, diploid females (chromosomes half irradiated, half not, cytoplasm irradiated). Fertilized eggs most seriously injured in respect to egg chromosomes develop into recessive, androgenetic, haploid males (chromosomes not x-rayed, cytoplasm x-rayed) (Whiting, 1946a). The present

paper is concerned primarily with the two types of males. X-ray induced changes common to both afford proof for cytoplasmic injury.

Eggs were separated according to time of laying into those irradiated in first meiotic metaphase (metaphase I) and those treated in first meiotic prophase (prophase I) (Whiting, 1945a).

For x-ray treatments a dual-tube self-rectifying outfit with a simultaneous cross-firing technic was used. The secondary voltage was 182 kv. and the tube current on each tube was 25 ma. The heavy glass of the tube walls and 5 mm. of bakelite of the tube shields gave the filtering value of 0.2 mm. copper shield. The output intensity was 7210 r per minute, distance 9.5 cm. Females were placed in gelatine capsules for treatment. All breeding was carried on at about 30° C. Lethal dose [2] is the lowest dose used after which no eggs have hatched.

When dose was fractionated, it was divided into three periods of approximately equal length. Two-hour intervals were allowed between exposures for experiments on hatchability, one-hour intervals for those on androgenetic males.

Observations

In order to demonstrate the significance of new data, some results, previously published, must be summarized briefly. Studies (Whiting, 1945a) on hatchability of 6824 eggs x-rayed in metaphase I have demonstrated that, when they are unfertilized, (a) the survival cure is exponential, (b) lethal dose is about 2400 r, and (c) hatchability is not changed significantly by variation of intensity of treatment, fractionation of dose or delay in oviposition. When these eggs are fertilized after treatment by untreated sperm their hatchability is not significantly changed. It was concluded, therefore, that death is due to dominant lethal effects which arise from single irreversible events. Eggs, irradiated in this stage, and fertilized by untreated sperm, may give rise to diploid females if dose is sub-lethal or to an occasional androgenetic male at either sub-lethal or lethal doses (Whiting, 1946b).

Cytological study (Whiting, 1945a) shows that chromosome fragments (terminal deletions?) may be present in the first meiotic division after treatment in metaphase I, that these fragments increase in number with increased dose, and that chromatin bridges (resulting from sister-chromatid union?) are present in the second meiotic division. These bridges are permanent and, since the egg pronucleus remains attached to them, it is pulled into a "tear-drop" as it moves inward. In unfertilized eggs, it undergoes cleavage and, in fertilized eggs, it usually contacts the male pronucleus and fuses with it. The diploid nucleus then divides and shows clearly the difference between the two groups of chromosomes, treated and untreated. In both cases, chromatin bridges are present in cleavage divisions and death of embryos ultimately occurs. Occasionally, however, the egg pronucleus is retarded to such a degree that the sperm pronucleus divides without it and a normal, haploid androgenetic male is formed (Whiting, 1948). Androgenesis results, then, from structural changes in the x-rayed maternal chromosomes of the type which, when less extreme, cause death to gynogenetic males and to females.

[2] Apparent inconsistencies in lethal doses in successive papers dealing with Habrobracon eggs are due to changes in method of calibration at the Marine Biological Laboratory. Conditions of treatment have not varied. In this paper all doses have been corrected for the latest measurements.

Hatchability studies on 12,634 eggs irradiated in prophase I (Whiting, 1945a) have demonstrated that, when they are not fertilized, (a) the dose-action curve is exponential only at lower doses and that, after higher doses, response increases at a disproportionate rate, (b) lethal dose is about 54,000 r, and (c) hatchability is not changed significantly by variation of intensity of treatment or delay in oviposition but is increased at high doses by fractionation of dose.

Prophase I eggs, free from dominant lethal changes after exposures to sub-lethal doses of x-rays, and fertilized by untreated sperm, develop into females. No androgenetic males have been produced by them. Cytological study of divisions after treatment in this stage has shown (Whiting, 1945b) that fragments, bridges, or both may appear in either meiotic division but that bridges, rarely present in the second meiotic division, are single and do not retard the egg pronucleus. No androgenetic males develop in these eggs, therefore, because of the absence of mechanical hindrance to free movement of the egg pronucleus. Even after very heavy treatments, eggs often fail to show any chromosome aberrations.

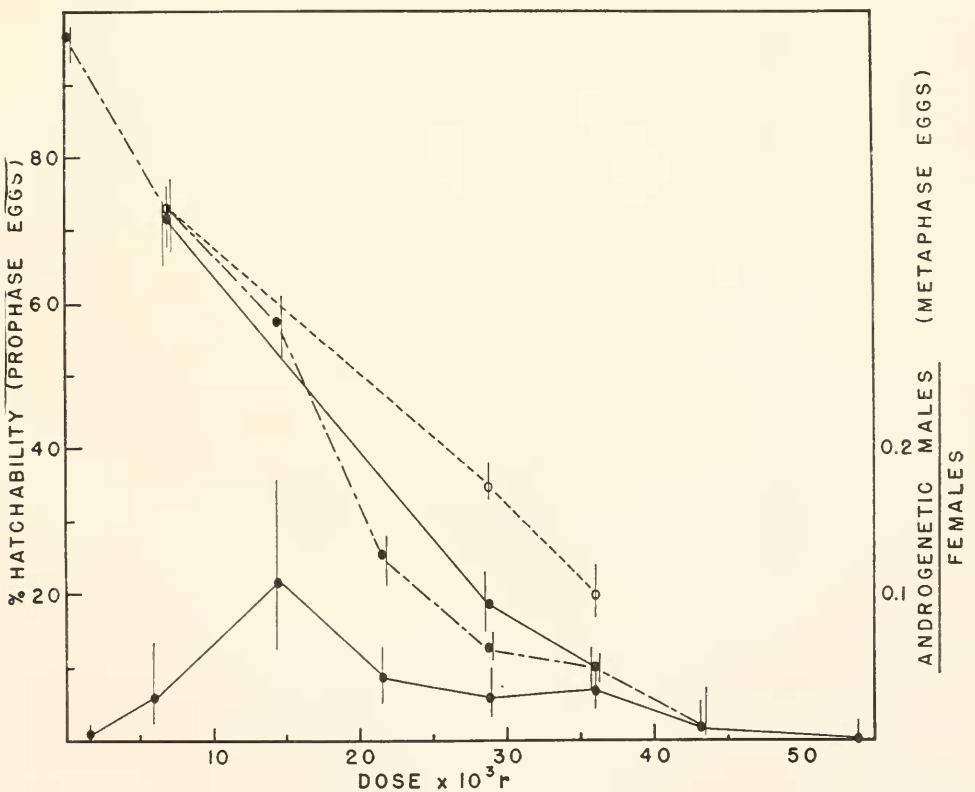

FIGURE 1. Lowest curve, $\frac{\text{androgenetic } ♂♂}{♀♀}$, plotted against dose. Remainder are dose-action curves for hatchability of unfertilized prophase I eggs. Continuous treatments (●), two experiments (—·—·—·) and (————); fractionated treatments (○), (– – – –). 95 per cent confidence interval is indicated for each experimental value.

Lowest dose after which significant reduction in hatchability was recorded was 50 r for metaphase I and 850 r for prophase I.

In Table I are assembled the data on the occurrence of androgenetic males in relation to dose. Results produced under constant experimental conditions are summarized in section A of the table and it is these which are expressed graphically in Figure 1. To be noted especially are the facts that (a) although androgenetic males develop only in eggs x-rayed in metaphase I (lethal dose for egg nucleus about 2400 r), they occur after treatment with doses up to that lethal for prophase I eggs

TABLE I

Androgenetic males in relation to dose and number of females x-rayed. A. Females from wild type stock No. 33, experimental conditions carefully controlled. B. Miscellaneous females, experimental conditions not controlled.

	Dose in r units	No. ♀ ♀ treated	No. androgenetic ♂♂	Androgenetic ♂♂ / ♀ ♀ Treated
A	675–2700	1015	5	0.0049
	6000	192	6	0.0313
	14,420	146	16	0.1095
	21,630	469	20	0.0426
	28,840	487	15	0.0308
	36,050	533	19	0.0356
	43,260	358	4	0.0111
	54,075	344	1	0.0029
		3544	86	0.0243
	64,169–144,200	359	0	
	64,169	181	0	
	64,890(frac.)	97	2	0.0206
		4000	88	
B	6000	65	1	
	12,000	150	4	
	14,420	623	6	
	40,000?	52	3	
	50,000?	89	0	
	50,000?(frac.)	87	8	
	Total	5066	110	

(lethal dose about 54,000 r), and (b) that androgenetic males per treated female increase with dose up to about 15,000 r and then decrease, and (c) that fractionation of dose permits development of androgenetic males at doses lethal to them when dose is administered continuously. Data on fractionation and androgenesis are not extensive but in each of two experiments one androgenetic male was produced in comparison with none from 181 females treated with continuous dose. In two experiments listed in section B a similar difference between eggs exposed to continuous and fractionated treatments was found. Dose was not accurately measured but was probably about 50,000 r.

The chance that the array of ratios in section A, column 4, is a random variation of uniformity in expectation is infinitesimal according to the χ^2 test ($P = 0.000000$).

Facts relevant to the discussion of cytoplasmic injury are expressed graphically in Figure 1. They fall into two categories, those related to hatchability of unfertilized eggs x-rayed in prophase I (x-rayed chromosomes in x-rayed cytoplasm) and those related to incidence of androgenetic males (untreated chromosomes in x-rayed cytoplasm). The 95 per cent confidence interval for each experimental value is plotted in the figure. Tables of confidence of Ricker (1937) and of Clopper and Pearson (1934) were used.

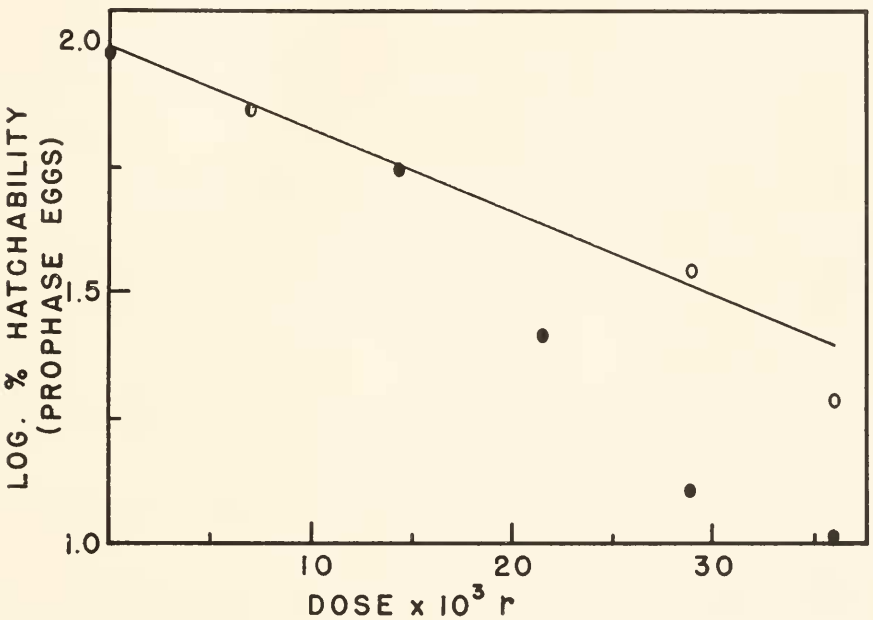

FIGURE 2. Percentages of hatchability of unfertilized prophase I eggs plotted semilogarithmically against dose; control and continuous treatments (●), fractionated treatments (○). By method of least squares a straight line was fitted to data of untreated, one minute (continuous + fractionated), two minutes (continuous), four minutes (fractionated) and five minutes (fractionated). For significance of experimental values consult Figure 1.

For hatchability of eggs x-rayed in prophase I with continuous treatment, there are two dose-action curves; with fractionated treatment, one. The former curves are not exponential, the latter is (Fig. 2). Note that the dose at which response to fractionation becomes apparent is about 15,000 r.

The curve representing androgenetic males per female rises to about 15,000 r and then falls until the lethal dose for them is reached. A steadily increasing number of chromatin bridges with resultant increase in retarded egg pronuclei is to be expected with increase of dose, so that androgenetic males should increase steadily with dose if there were not a concomitant increase of some factor which reduces their viability. Since this factor is x-ray induced, it must be cytoplasmic.

Several questions may have occurred to the reader and these are discussed at this point.

Why are there so few androgenetic males, even at doses optimal for their occurrence? Is there a high mortality of androgenetic embryos and, therefore, some permanent cytoplasmic injury at all doses? A comparison of androgenetic males per metaphase I eggs laid (6/381) with androgenetic cleavage per metaphase I eggs studied cytologically (6/291) shows that the difference between these two groups is not significant at the dose ranged used, 14,420 – 28,840 r. The small number of androgenetic males is due, then, not to the death of many but to the fact that very few egg pronuclei are sufficiently retarded to permit development of sperm nuclei alone (Whiting, 1948).

How do androgenetic males compare in vigor, viability, fertility and mutation rate, with gynogenetic males produced by the same females and with untreated controls? Detailed data on this subject are being prepared for publication, but in summary it can be stated that there is a striking difference between androgenetic males and gynogenetic males from the same females. All but one of the 110 androgenetic males found have been perfectly normal, vigorous, and fully fertile. The single exception died as a pupa. No visible mutations have appeared in them. They are indistinguishable from untreated controls in every way. Gynogenetic males tend to die as larvae or pupae and survivors have shown a significantly higher percentage of visible mutations, 29 among 417 or 6.95 per cent. Using the exact method of treating contingency tables (Yates, 1934), it is found that the chance that these percentages, 0 and 6.95, are variants of uniformity in expectation is very low ($P = 0.00188$).

Do heavily irradiated females lay fewer eggs than those treated with lighter doses? If so, this would reduce ratio of androgenetic males to mothers. Detailed analysis (Whiting, 1945a) showed that, after any dose up to 150,000 r, the average number of metaphase I eggs per female is the same as for controls.

Do sperm enter heavily irradiated eggs as freely as controls and do they move about in the egg normally? X-rayed females mate readily after any dose and, after sub-lethal doses, produce the expected ratio of daughters.

Are the cytological phenomena of the x-rayed egg nucleus the same at very high doses as at low? No accurate counts have been made of relative numbers of fragments, bridges, retarded pronuclei, etc., at very high dose but all these conditions have been observed in many eggs after treatment of metaphase I with 60,000 r.

Discussion

Evidence for chromosomal injury as the cause of death of Habrobracon eggs irradiated in metaphase I, with doses up to 2400 r, is convincing. The survival curve is exponential, and cytological study has demonstrated that ootids without chromatin fragments also give an exponential dose-action curve (Whiting, 1945a; Lea, 1946). This curve is higher than actual hatchability and suggests that some ootids without fragments are inviable. This can be explained by failure to see small fragments in some eggs. Such a failure is understandable in view of the small size of Habrobracon chromosomes and, therefore, still smaller size of the fragments and the granular nature of the yolk in which they lie. It is highly probable that each egg that fails to hatch has at least one fragment in it.

That death of x-rayed metaphase I eggs is due to chromatin loss through terminal deletions which act as dominant lethals (Muller, 1940; Pontecorvo, 1941) has been accepted by Lea (1946) and he discusses the similarity of these results to those of Sonnenblick (1940) on Drosophila eggs. Lea writes, after a detailed discussion of the subject (1947), "It is evident that dominant lethals in unfertilized eggs, as well as in the sperm, can be explained by lethal types of structural change." From the data on hatchability of x-rayed metaphase I eggs of Habrobracon, he estimated that there are 1.7 breaks primarily produced per 1000 r per haploid chromosome complement and that all breaks are permanent (Lea, 1946). This accounts for the extreme sensitivity of these cells by a method consistent with facts and with theories accepted for the explanation of similar responses of sperm cells to x-rays.

Evidence for causes of death of Habrobracon eggs irradiated in prophase I, with doses up to 54,000 r, suggests that both chromosomal and cytoplasmic changes are involved. The study of the nature of chromosomal injury is complicated by the type of dose-action curve which is exponential only for the first three points (including controls) (Fig. 2). There is no response to fractionation at these doses. Eggs, exposed to lethal doses, have been seen with chromosomal aberrations although many seem to lack them. This does not preclude their presence, however. The conditions of the chromosomes at time of treatment would allow for many types of chromosomal rearrangements, and their identification is more difficult than that of the relatively large fragments seen after irradiation in metaphase I. The increased response of x-ray prophase I eggs to higher doses suggested induction of a new phenomenon at about 15,000 r. This was interpreted (1945a) as due to an increase in multiple-hit, complex rearrangements which would increase rapidly at higher doses (Sax, 1938) and complicate the curve. Many types of multiple-hit chromosomal changes are viable, however, and would not be expected to reduce hatchability to the extent indicated in these experiments.

If the rise in mortality of both androgenetic and gynogenetic males induced by doses above 15,000 r were chromosomal in nature, it would be necessary to postulate that injury (breaks) in metaphase chromosomes decreases while that in prophase chromosomes increases above that point in response to increased dose. There remains the possibility that stickiness of chromosomes replaces breaks but this would increase androgenesis since it is permanent after high doses in the forms in which it has been reported (Sax, 1942). It is unlikely to occur in prophase chromosomes. There is no evidence for it in Habrobracon eggs irradiated with doses up to 60,000 r. If cytoplasmic injury is not involved it would also be necessary to suppose that fractionation of dose increases injury in metaphase chromosomes but decreases it in prophase chromosomes. One-hit chromosomal breaks (terminal deletions?), characteristic of metaphase injury, do not respond to fractionation of dose.

The first appearance of change in response to dose at about 15,000 r, its effect on reducing survival of gynogenetic and androgenetic males, its response to fractionation in increasing both types of males, combine to indicate that this change is due to cytoplasmic injury.

In Figure 2 are plotted semi-logarithmically percentages of hatchability of unfertilized x-rayed prophase I eggs. By the method of least squares a straight line was fitted to the data giving hatchability of untreated, one minute (continuous and frac-

tionated), two minutes (continuous), four minutes (fractionated) and five minutes (fractionated) of treatment. For the last, percentage of hatchability is below expectation on the basis of one-hit permanent changes. One fraction of treatment at this dose was longer than any others used and perhaps this reduced recovery. Curve for hatchability after continuous treatments at high doses is clearly not exponential. All data suggest that, if proper conditions of fractionation are found, a good exponential curve of hatchability can be obtained which will represent the effects of dominant one-hit irreversible chromosomal changes only since cytoplasmic injury will be prevented. Under such conditions, the number of androgenetic males should increase steadily with increase in dose and percentage of visible mutations should be the same for both continuous and fractionated treatments. Lethal dose for prophase chromosomes should be over 90,000 r.

X-ray induced chromosomal changes have been explained by some investigators as "direct-hit" changes due to the production of ionization in particular molecules and this is known as the "target theory" (Giese, 1947; Lea, 1947). Criteria of the validity of this theory are (a) independence of response to change in intensity or to fractionation, (b) absence of a threshold for response, (c) dependence of response or wave length and (d) an exponential survival curve (for one-hit aberrations). Results of irradiation of metaphase I eggs clearly support this theory. Unfortunately, no experiments have been carried out with radiations of different wave lengths.

When cytoplasmic injury is eliminated by fractionation at high doses, the resultant phenomena likewise support the target theory of chromosomal change in eggs x-rayed in prophase I.

In contrast to chromosomal change, cytoplasmic injury has a rather high and definite threshold.

Does the cytoplasmic injury in itself prevent the androgenetic embryo from maturing or does it influence the untreated chromosomes so that they are incapacitated? Untreated male pronuclei in cytoplasm x-rayed with 70,000 r look normal. The consistent absence of any injury in surviving androgenetic males favors the view that cause of death of the embryo is directly cytoplasmic.

Two facts have been noted about heavily irradiated eggs: (1) when laid they are softer and more flexible than control eggs and (2) the first cleavage nucleus, whether haploid or diploid, tends to be situated more posteriorly than in control eggs. These facts suggest decrease in cytoplasmic viscosity.

To this author, the situation which exists at four minutes of treatment, 28,840 r, represents an ideal one for testing effects of environmental changes on cytoplasmic injury and thereby, perhaps, obtaining some clue as to its nature. Any response similar to that of fractionation of dose would indicate reduction of cytoplasmic injury.

Summary

Wild type Habrobracon females were x-rayed and mated to untreated recessive males. Two kinds of haploid males were produced, gynogenetic (x-rayed chromosomes in x-rayed cytoplasm) and androgenetic (untreated chromosomes in x-rayed cytoplasm).

Eggs x-rayed in metaphase I × untreated sperm

Gynogenetic males develop from the unfertilized eggs, androgenetic males from fertilized. Death of the former and origin of the latter are caused by different degrees of the same type of x-ray induced chromosomal injury. Dose-hatchability curve for gynogenetic males is exponential and their lethal dose is that of the chromosomes in this stage, about 2400 r. Percentage of androgenetic males increases up to about 15,000 r, then gradually decreases until none is produced at about 54,000 r which is the lethal dose for the cytoplasm in this stage. Percentage of androgenetic males can be increased at doses above 15,000 r by fractionation.

Eggs x-rayed in prophase I × untreated sperm

Gynogenetic males develop from the unfertilized eggs. No androgenetic males develop due to absence of type of chromosome aberration necessary for their formation. Dose-hatchability curve for gynogenetic males is exponential up to about 15,000 r, after which it falls at an increased rate. It can be restored to an exponential curve by fractionating dose. Lethal dose is about 54,000 r. This is the lethal dose for cytoplasm. That for the chromosomes in this stage is considerably higher.

Chromosomal vs. cytoplasmic injury

Some androgenetic males survive after dose over twenty times greater than that which is lethal for chromosomes of eggs in which they develop. At all doses they resemble the controls and differ significantly from gynogenetic males in visible mutation rate, viability, and fertility. The changes peculiar to gynogenetic males must be chromosomal in origin since both types of males develop in irradiated cytoplasm. Evidence suggests that these changes are directly induced and supports the target theory of chromosomal injury.

Since there is no evidence for chromosomal injury in surviving androgenetic males, the reduction of their number at doses above 15,000 r, through embryonic death, must be directly cytoplasmic.

The increase in survival of both gynogenetic and androgenetic males in response to fractionation of dose must be due to reduction of cytoplasmic injury since they have only x-rayed cytoplasm in common.

Conclusion

At doses from 50 r to about 15,000 r, death of Habrobracon eggs (one stage more sensitive than sperm, the other less so) is due to chromosomal injury. It is not reduced by fractionation of dose or changes in intensity.

Above 15,000 r, x-rays induce cytoplasmic injury which may exert a lethal effect on developing embryos. It is reduced or prevented by fractionation of dose.

Injured cytoplasm has an "all or none" effect. It may kill embryos but does not induce visible mutations in untreated chromosomes or reduce fertility or viability of survivors. Its expression resembles, therefore, dominant lethal genetic effects and it acts directly in killing the embryo and not indirectly through injury to untreated chromosomes.

Evidence supports the target theory of chromosomal injury.

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